I. AMENDMENTS TO THE SPECIFICATION

Please amend the specification as follows:

Please delete the "Brief Description of the Drawings" beginning at page 7, line 1 through page 12, line 2. See Attachment A, Application pages 7-12.

Please amend the paragraph beginning on page 12, line 18 through page 13, line 21 of the specification, as follows:

In an attempt to clone the carp NT-6, two exact oligonucleotides representing the amino acid sequence YSVCDS (GTACTCTGTGTGTGACAG) SEQ ID NO.: 3 & 4 and INAACV (CA CACATGCAGCGTTGA) SEQ ID NO.: 5 & 6, which corresponded to two conserved regions of NT-6, were designed ~~(Figure 1)~~. Carp genomic DNA (0.6 μg) was used in the polymerase chain reaction (PCR) as template. One-tenth of the reaction was further amplified by the same set of primers in a second PCR. The fragment was then gel purified and the ends were blunted by KLENOW™ (Amersham, UK) before being ligated to *SmaI*-cut pBLUESCRIPT™ (Stratagene, CA). After transformation into XL-1 BLUE™, the plasmid was purified and double-stranded DNA sequencing was performed (Sanger et al., 1977). The resulting fragment was designated as NT-7. Rapid amplification of cDNA ends (RACE) was employed in order to clone the full-length NT-7 from carp skin RNA. Methods were the same as stated in the protocol (GIBCO®, Life Technologies, NY). The 5'- and 3'- RACE revealed the sequence of part of the pre-pro region and the 3'-untranslated region. To clone the entire mature region, two primers (AAATGATACG GGGAGCC (SEQ ID NO.: 7) and AAGGGGCGGAGTCTCAG (SEQ ID NO.: 8)) located at the pre-pro and 3'-untranslated regions, respectively, were used to amplify NT-7 from 1.3 μg of carp genomic DNA using VENT™ polymerase (New England BioLabs, MA). The resulting fragment of 553 bp was subcloned into pBLUESCRIPT™ by blunt-end ligation as mentioned above. The partial sequence of carp NGF was amplified by PCR from 0.3 μg carp genomic DNA by a pair of degenerate primers that corresponded to the conserved regions FYETTC (SEQ ID NO.: 13) and ACVCV (SEQ ID NO.: 14). To clone the full-length *Xiphophorus* NGF and NT-6, two pairs or primers (CTTAGATCGTGTGCCCATG (SEQ ID NO.: 9) and GGGTGAGTCTTCAATGCTG (SEQ ID NO.: 10) for NGF; ATAACGTGGACGTGTGCCC (SEQ ID NO.: 11) and CAAGAGC

GGTCCACACCTC (SEQ ID NO.: 12) for NT-6) were designed and PCR was performed using 1.8 μg *Xiphophorus* genomic DNA as template. The resulting products were subcloned into the expression vector pMT21. The cDNA sequence for carp NT-7 has been submitted to GENBANK™, with the Accession Number of ~~Q94949~~ U94949.

Please amend the following paragraph beginning on page 14, line 24 through page 15, line 5 of the specification, as follows:

For RT-PCR analysis, 2 μg of RNA from various carp tissues was used as template in the reverse transcription that utilised SUPERSCRIPT RT™ (Gibco, NY). The RNA was pretreated with 1 unit of RNase-free DNase (Promega, WI) for 30 minutes at 37°C. For the control, no RT was added and was substituted by DEPC-H_2O. One tenth of the reaction was amplified by two primers flanking the insertion ~~(Figure 1)~~. The resulting product was analysed in agarose gel and subjected to Southern blotting, using the same fragment used in the Northern blot analysis as probe.

Please amend the following paragraph beginning on page 18, line 19 through page 19, line 5 of the specification, as follows:

A DNA fragment of 340 bp was amplified from carp genomic DNA using primers corresponding to two conserved regions of NT-6 ~~(Figure 1 A)~~. Subsequent cloning and DNA sequence analysis suggested the fragment might represent partial sequence of a novel neurotrophin. Northern blot analysis revealed its relatively strong expression in skin (see below). Therefore, in order to obtain the full-length sequence, 5'- and 3'- RACE (rapid amplification of cDNA ends) was performed using carp skin cDNA as template. The full-length clone which contained the putative translation start codon ATG and the signal peptide was not generated by the 5'-RACE. However, DNA sequence of the entire mature region could be obtained from the resulting clones. Subsequently, PCR using primers designed from sequence at the pre-pro and the 3' -untranslated region was performed to amplify the entire mature region.

Please amend the following paragraph on page 19, lines 7-18 of the specification, as follows:

DNA sequence analysis revealed that the pre-pro region was terminated at the two basic amino acids, arginine, followed by a mature region of 133 amino acids ~~(Figure 1-A)~~ SEQ ID NO.: 1). The R-X-K/R-R sequence was conserved in all neurotrophins and represented the proteolytic cleavage site, at which the mature protein was cleaved from its larger precursor (Hosaka et al., 1991). The presence of all 6 conserved cysteine residues together with the nearby conserved regions in the mature protein suggested that it represented a novel neurotrophin molecule. Amino acid alignment of this novel neurotrophin, designated NT-7, with that of *Xiphophorus* NGF and NT-6 revealed 66% identity ~~(Figure 2A)~~. Moreover, alignment with carp BDNF, chick NT-3, and *Xenopus* NT-4 suggested even more distant relationships ~~(Figure 2A)~~. However, NT-7 lacked some amino acid residues which were conserved in all NGF molecules identified so far ~~(Figure 2B)~~.

Please amend the paragraph beginning on page 19, line 20 through page 20, line 8 of the specification, as follows:

Because of its structural similarity to NGF, it was possible that NT-7 merely represented the carp homologue of NGF. However, Southern blot analysis of carp genomic DNA revealed that different DNA fragments hybridised with *Xiphophorus* NGF and carp NT-7 (data not shown), suggesting that NGF and NT-7 represented different genes. To clone the carp NGF homologue, PCR was performed using several pairs of degenerate primers that corresponded to conserved regions near the cysteine residues. As a result, a 177-bp fragment was amplified from carp genomic DNA by one of the pairs of primers ~~(Figure 1-B)~~. Analysis of the deduced amino acid sequence indicated its close resemblance to NGF; it shared amino acid identity to the corresponding region of *Xiphophorus* NGF, carp NT-7, *Xiphophorus* NT-5, chick NT-3, carp BDNF, and *Xenopus* NT-4, respectively ~~(Figures 2A and 2B)~~. In addition, it lacked those amino acid residues that were highly conserved in either BDNF, NT-3, or NT-4 of different species (Hallbook et al., 1991). These finding strongly suggested that this clone represented the partial sequence of carp NGF.

Please amend the following paragraph on page 20, lines 13-21 of the specification, as follows:

To examine whether NGF, NT-6, and NT-7 represent three different genes that coexist in one genome, Southern blot analysis of *Xiphophorus* genomic DNA, digested by *Hind*III, was performed and hybridised with *Xiphophorus* NF, NT-5, or carp NT-7 cDNAs under low stringency conditions. It was found that different patterns were obtained by hybridisation with the three neurotrophin cDNAs. The DNA fragments that hybridised with NGF were ~8 and ~3 kb, while a single fragment of ~8 and ~2 kb hybridised with NT-6 and NT-7, respectively ~~(Figure 3)~~. This suggested that NGF, NT-6, and NT-7 did represented three different genes.

Please amend the paragraph beginning on page 21, lines 1-5 of the specification, as follows:

The spatial expression of NT-7 in adult carp was studied by Northern blot analysis. A single transcript of about 1.1 kb was detected in skin and heart, though weak expression was also found in brain and intestine ~~(Figure 4A)~~. This was in contrast with that of NT-6, where expression in adult fish was predominantly found in brain, gill, liver, and eye, but not skin (Gotz et al., 1994).

Please amend the paragraph on page 21, lines 7-10 of the specification, as follows:

Furthermore, the expression of NGF was studied in adult *Xiphophorus* in order to compare its spatial expression with that of NT -7. Northern blot analysis revealed dominant expression of a single transcript of ~3.6 kb in eye and gill, though weak expression could be detected in skin ~~(Figure 4B)~~.

Please amend the paragraph beginning on page 21, line 17 through page 22, line 5 of the specification, as follows:

One of the interesting features of NT -7 was the insertion of 15 amino acids between the second and third cysteine residues, a feature not found in any other known neurotrophins (including *Xiphophorus* NT -6 which contained an insertion of 22 amino acids at the corresponding position). The insertion contained only 4 glycine and 2 basic amino acid residues compared to the 8 glycine and 6 basic amino acid residues in *Xiphophorus* NT-6 that corresponded to the heparin-binding domain. In order to rule out the possibility of the insertion

being an intron, RT-PCR was performed using a pair of primers flanking the insertion. All the tissues that were shown to express NT -7 in the Northern blot produced a single band which corresponded to the size having the insertion. Moreover, the band was absent when RT was performed using the same RNA samples but without reverse transcriptase ~~(Figure 5)~~. Thus, the resulting product which contained the insertion was not originated from contaminating genomic DNA, and there was no detectable alternative form of the NT -7 transcript that lacked the insertion.

Please amend the following paragraph on page 23, lines 4-20 of the specification, as follows:

All the expression constructs were transiently transfected into COS-5 cells and conditioned medium was collected after 3 days. Neurite outgrowth assay of chick embryonic dorsal root ganglia (DRG) was employed to test the biological activities of the various neurotrophins. Addition of either *Xiphophorus* NGF or NT-7 resulted in robust neurite outgrowth from E8 DRG ~~(Figure 6)~~. Moreover, NT-7(D15) also showed comparable activity, suggesting that NT-7 was still active without the 15-amino-acid insertion. In contrast, the effect of either NT-6 or NT-6(D22) was similar to that obtained from conditioned medium of mock-transfected cells. Similar results were observed for the two NT-6 constructs with pre-pro exchange (data not shown). The neurotrophic activity of NT-7 was further investigated by the survival assay of dissociated DRG neurons. Consistent with the results of the neurite outgrowth assay, both NT-7 and NT-7(D15) could support the survival of E8 chick DRG neurons ~~(Figure 7)~~. On the other hand, NT-6 and NT-6(D22) failed to promote significant neuronal survival, irrespective of the pre-pro region. Thus, the neurotrophic activity of NT-7 was demonstrated by its ability to stimulate neurite outgrowth and survival of DRG neurons, in a manner similar to that induced by *Xiphophorus* NGF.

Please amend the paragraph on page 24, lines 1-17 of the specification, as follows:

To elucidate its mechanism of action, the interaction between NT-7 and the various Trk receptors was studied by phosphorylation of different Trk receptors ectopically expression in fibroblasts. Because of its structural similarity to NGF, it would be expected that NT-7 showed receptor specificity to TrkA. Indeed, compared with the conditioned medium of mock-transfected COS cells, NT-7, NT-7(D15), and NT-6(D22) could weakly phosphorylate TrkA but

not TrkB or TrkC ~~(Figure 8)~~. It should be noted that despite the relatively weak level of TrkA phosphorylation, it was consistently observed in triplicate experiments. The extent of TrkA phosphorylation elicited by NT-7, NT-7(D15), and NT-6(D22) was considerably lower than that obtained by *Xiphophorus* NGF, NT-6(D22) failed to promote neurite outgrowth and survival of DRG, yet its induction of TrkA phosphorylation was similar to NT-7 and NT-7(D15). On the other hand, with the presence of the 22-amino-acid insertion, NT-6 was unable to stimulate any detectable level of TrkA phosphorylation, probably because of its binding to the cell surface or extracellular matrix, which subsequently hindered its release to the conditioned medium. Similarly, NT-6(D22) with NGF pre-pro region could induce weak TrkA phosphorylation (data not shown).

Please amend the paragraph page 25, lines 9-17 of the specification, as follows:

It was found that only NGF-Fc could stimulate the neurite outgrowth of DRG, while the activity of NT-6(D22)-Fc as well as NT-6-Fc (data not shown) was similar to that of conditioned medium of mock-transfected cells ~~(Figure 9)~~. Therefore, the different response of DRG to the two neurotrophins in our study was likely due to the lower potency of NT-6 in promoting neurite outgrowth. Indeed, the EC50 of purified NT-6 in supporting the survival of chick DRG was quite high (about 100 ng/ml; Gotz *et al.*, 1994). It was therefore possible that the expression level of NT-6 and NT-6(D22) in our study was not high enough to reveal its activity on DRG neurons.

Please amend the paragraph beginning on page 25, line 22 through page 26, line 21as follows:

NT-6 was originally cloned from the aquarium fish *Xiphophorus maculatus* (Gotz et al., 1994), but so far no homologue in any other vertebrate was found. With the assumption that NT-6 was highly homologous among different types of fish, particularly at the conserved regions around the cysteine residues, PCR was performed using a pair of primers at two conserved regions in order to clone the carp NT-6 homologue. It was therefore surprising that the resulting fragment, designated NT-7, only shared 66% amino acid identity to *Xiphophorus* NT-6. Apart from the relatively low percentage of homology, there was also considerable differences between the primary structure of the two neurotrophins. For example, the lack of an amino acid between Asn23 and Lys24, which was characteristic to NT-6 but not any other known neurotrophins, was

found in NT-7 ~~(Figure 2A)~~. Moreover, the insertion present in NT-7 was considerably different from that of NT -6 in terms of the length and number of basic amino acid residues. Southern blot analysis demonstrated that NGF, NT-6, and NT-7 represented three different genes in the *Xiphophorus* genome. The partial sequence of carp NGF cloned in our study indicated that NGF and NT-7 were indeed two different genes. Since the homology between NGF and NT-7 is very similar to that between NT-7 and NT-6 (about 66 % in both cases), it supports our claim that NT-7 represents a different gene from NT-6, rather than the carp NT-6 homologue. In addition, the spatial expression of NT-7 in adult tissues was quite different from that of *Xiphophorus* NGF and NT-6. In particular, high level of expression of NT-6 was reported in adult brain, eye, and gill, while NT-7 expression in the brain was low, and was even undetectable in eye and gill. Taken together, despite the relatively high evolutionary rate of neurotrophin in lower vertebrates (Gotz *et al.*, 1992), NT-7 represents a novel member of the neurotrophin family.